Review

NR4A1, 2, 3 – an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis

Q.-X. Li, N. Ke, R. Sundaram and F. Wong-Staal

Immusol, Inc. San Diego, CA, USA

Summary. Three members of the NR4A1/Nur77/ NGFIB orphan nuclear hormone receptor subfamily (NR4A1, NR4A2, and NR4A3) belong to the steroid nuclear hormone receptor superfamily. They share similar structural features and have no known natural ligand. They constitute immediate early genes that are induced by serum, growth factors and receptor engagement and are thus implicated in cell mitogenic responses. These nuclear receptors are transcription factors that exert their functions through activation and subsequent induction of the downstream pathways. They have been shown to play a role in complex pathways of cell survival and apoptosis. Although the expression of these genes have been shown to be pro-survival, it has also been reported that NR4A1 expression can cause apoptosis. These two opposite effects apparently result from distinct mechanisms: either transcriptional activation of genes responsible for cell survival or cell apoptosis, or translocation into the cytoplasm where they target the mitochondria and cause cell apoptosis via Bcl-2 binding. The latter mechanism constitutes a new paradigm of cellular apoptosis. In vitro functional studies using over-expression (gain of function) or gene inactivation (loss of function) type assays, combined with transgenic or knockout animal data in vivo, have revealed these effects and their physiological roles, including thymocyte development for NR4A1/3 and pro-survival in CNS for NR4A2. Recent studies have also suggested an important role of these receptors in cell transformation and tumorigenicity via both their anti-apoptotic and pro-apoptotic functions. In particular, the recent identification of a functional ligand for NR4A1 suggests that these members could potentially serve as drug targets for disease indications such as cancer. While many aspects of these receptors have been previously reviewed, this article focuses on new experimentation and discovery of their apoptotic and carcinogenic roles, and discusses their potential roles as therapeutic targets.

Key words: NR4A1, NR4A2, NR4A3, Apoptosis, Transformation, Carcinogenesis

Nur77/NR4A1 nuclear hormone receptor family

NR4A1, NR4A2 and NR4A3 are three members of an orphan nuclear hormone receptor family referred to as Nur77 family. NR4A1 is also called Nur77, TR3, or NGFI-B; NR4A2 is also named as Nurr1, RNR-1 or TONOR; while NR4A3 has also been called Minor and Nor-1. They are all transcription factors belonging to the superfamily of steroid nuclear hormone receptors (Forman et al., 1995; Mangelsdorf et al., 1995; Enmark and Gustafsson, 1996). The structures of these orphan receptor proteins have been reviewed in detail elsewhere (Hsu et al., 2004); they include an activation domain at their N-terminus (A/B or AF-1), a DNA binding domain (C or DBD) (Wilson et al., 1991) and a ligand binding domain (E, LBD, or AF-2) at their C-terminus (Enmark and Gustafsson, 1996) (Fig. 1, Table 1). NR4A1, 2 and 3 are highly homologous in the DBD domain (~91-95%), modestly homologous in the LBD domain (~60%), and very divergent in their activation domain (Table 1). All three members are found in the nucleus since their DBDs also contain nuclear localization signals (NLS) (Katagiri et al., 2000). The LBD of NR4A1 contains three nuclear export signals (3xNESs) as well as a Bcl-2 binding domain (Table 1) (Katagiri et al., 2000; Lin et al., 2004). The DBD's bind to the consensus DNA sequence AAAGGTCA (NBRE, NGFI-B response element) as monomers, and to AAAT(G/A)(C/T)CA (NurRE, Nur-responsive element) by homodimerization or heterodimer interactions with each other to activate different target gene expression (Perlmann and Jansson, 1995; Zetterstrom et al., 1996; Maira et al., 1999). Dimers show stronger activity over monomers, and different dimers exhibit different preferences for targeted DNA sequences. Nur77 family proteins are involved in a wide variety of biological processes, especially the ones involved in cell survival and apoptosis. They are immediate early genes induced by serum, growth factors and receptor engagement (Winoto and Littman, 2002).

Crystal structures of the functional domain of the members have been described, e.g. the DNA binding domain of NR4A1 complexed with its target DNA (Meinke and Sigler, 1999), and the LBD of NR4A2 and NR4A4, NR4A4 being the Drosophila ortholog of NR4A1, 2 and 3 (Baker et al., 2003; Wang et al., 2003). The crystal structure of the LBD of NR4A2 and their Drosophila ortholog NR4A2 are similar to the agonist-bound, transcriptionally active LBDs of other nuclear hormone receptors. The "classical" ligand-binding pocket on LBD is tightly packed with side chains from several conserved bulky hydrophobic residues (Wang et al., 2003). NR4A2 lacks the classical binding site for co-activators. However, the LBD of NR4A2 can still be regulated in mammalian cells, and its transcriptional activity is correlated with the NR4A2 LBD adopting a more stable conformation. Unlike other steroid receptors, which are constitutively expressed and their activity modulated by steroid ligands, the expression of NR4A1, 2 and 3 appears to be regulated by external stimuli through their expression. The regulation of NR4A1 transcription has been extensively reviewed (Zetterstrom et al., 1996; Winoto and Littman, 2002; Hsu et al., 2004).

Although these receptor transcription factors are considered to be orphan, they can be regulated by many hormones via the formation of heterodimers with other hormone receptors that do bind hormone ligands. These receptors can also influence the target genes of other nuclear receptors via formation of similar heterodimers. Furthermore, a recent study revealed that NR4A1 can directly bind to synthetic ligands and thereby modulate its activities (see below, (Chintharlapalli et al., 2005)).

NR4A1/2/3 receptor family in cell survival and apoptosis

Expression of these three receptors in tissues and organs varies, which may imply diverse physiological functions (Table 2). NR4A1 is widely expressed, but primarily in thymus, osteoblast, liver, and pituitary gland (Zetterstrom et al., 1996; Hsu et al., 2004). NR4A2 is expressed in the developing and adult central nervous system (Law et al., 1992; Paulsen et al., 1995; Le et al., 1999). NR4A3 mRNA was detected at low levels in the adult heart and skeletal muscle as well as in the fetal brain. It is expressed at high levels in the -pituitary gland. The expression kinetics of NR4A3 is similar to that of NR4A1 in activated T cells, and they appear to play partially redundant roles in T cells and adrenal glands (Cheng et al., 1997; Fernandez et al., 2000). This putative redundancy may explain the complete lack of phenotype in NR4A1 knockout animals. Transgenic mice (Calnan et al., 1995; Weih et al., 1996) and knockout mice (Cheng et al., 1997; Zetterstrom et al., 1997; Castillo et al., 1998; DeYoung et al., 2003; Hsu et al., 2004) have also been used to investigate the roles of these receptors *in vivo*. The phenotypes observed in some transgenic and knockout animal models are summarized in Table 3.

NR4A1, 2, and 3 have all been previously implicated in cell growth/survival and apoptosis. These functions appear to be derived from three biochemical properties of these receptors: 1) as transcription factors whose activation up-regulates target gene expression leading to cell proliferation and survival (Bras et al., 2000; Suzuki et al., 2003; Ke et al., 2004); 2) as transcription factors whose activation up-regulates target genes leading to cell apoptosis (Zhang et al., 1999; Chintharlapalli et al., 2005); 3) translocation to the cytoplasm and targeting mitochondria, thus triggering apoptosis (Li et al., 2000; Lin et al., 2004). These pro-survival and anti-survival biological functions in cells have been shown to play important physiological as well as pathological roles.

A variety of experimental tools have been used to study the biological functions and biochemical properties of the Nurr1 family of receptors. Over-expression (gain-of-function) (Bras et al., 2000; Suzuki et al., 2003), dominant negative mutants (Suzuki et al., 2003), antisense (Zhang et al., 1999) and more importantly, the recent RNAi-mediated gene-silencing (loss-of-function) (Kolluri et al., 2003; Ke et al., 2004; Chintharlapalli et al., 2005; Li et al., 2005) technologies have been broadly used to study the *in vitro* functions of these receptors. Since these receptors are transcription factors that share recognition sequences, these sequences can be used to generate reporter systems to investigate their activity. The downstream gene transcription activities also provide ways to explore the biological functions of these receptor molecules. Several types of

Table 1. Summary of the functional domains of Nur77 family.

DOMAIN	DESIGNATION	CONSERVATION	FUNCTION
A/B	AF-1	Low (21-27%)	Transactivation
C	DBD	High (> 90%)	zinc finger motif, DNA binding, 2x NLSs
D	Variable hinge		
E	LBD, AF-2	Intermediate (~ 60%)	Transactivation, 3x NESs, Bcl-2 binding
F			unknown

AF-1: Activation function-1C-domain function: DNA binding; LBD: ligand binding domain, dimerization domain, region, nuclear localization domain; AF-1: activation function-1; DBD: DNA binding domain contain NLSs (nuclear localization signals); NESs: nuclear export signals for NR4A1.

reporter systems have been shown to be extremely useful in exploring the biochemical and biological roles of these receptors. These includes:

1) NurRE-luciferase (or other reporter) can be used to assess transactivation activity (Ke et al., unpublished observations) (Fig. 1);

2) GAL4-NR4A1 (LBD)-luciferase can be used for screening functional binders for NR4A1 (Chintharlapalli et al., 2005) (Fig. 1);

3) GFP-TR3 fusion protein can be used for TR3 localization/translocation upon treatment (Lin et al., 2004) (Fig. 1).

NR4A1

Pro-survival role of NR4A1

NR4A1 is the most well studied member among this receptor family. Its expression can be induced by a number of different stimuli, e.g. stress stimuli like TNF (Suzuki et al., 2003), serum, and growth factors including NGF and T-cell receptor mediated signaling. Over-expression of NR4A1 was shown to prevent ceramide-induced cell death in a mature B-cell lymphoma (A20) (Bras et al., 2000), suggesting a pro-survival or anti-apoptotic function for NR4A1. However, this over-expression did not antagonize FasL induced apoptosis in the same cells, suggesting that the observed anti-apoptotic function does not involve Fas-mediated apoptosis pathway. Others have also reported that over-expression of NR4A1 in certain human lung cancer cells caused resistance to retinoic acid treatment and contributed to proliferation and neoplastic transformation (Wu et al., 1997).

In a subsequent study, the ectopic expression of NR4A1 in mouse embryonic fibroblasts (MEF) was shown to antagonize TNF induced apoptosis while a negative dominant mutant defective in the transactivation domain accelerates TNF induced cell death (Suzuki et al., 2003). This once again indicates

that NR4A1 is a survival effector in the TNF survival pathway. Furthermore, both caspase 3 and caspase 8 activities were reduced in cells over-expressing NR4A1, indicating the involvement of these two apoptosis enzymes. Additionally, NR4A1 was localized to the nucleus throughout these observations, indicating that its role as a transcription factor probably contributes to this effect.

NR4A1 silencing mediated by siRNA leads to a drastic reduction in cell growth/survival, as we have demonstrated in many cancer cell lines (Table 4). The reduced growth correlated well with increased levels of apoptosis. For example, transient transduction of cancer cells with lentiviral RNAi vector caused apoptosis (Ke et al., 2004) (Table 4). Consistent with our observation, the mitogenic function of NR4A1 is also observed in lung cancer cells (Kolluri et al., 2003). Therefore, the pro-

Table 2. Normal tissue expression patterns of NR4A1,2,3.

	NR4A1	NR4A2	NR4A3
Thymus	+	+	+
Brain		+	
Osteoblast		+	
Liver	+	+	
Pituitary gland	+	+	+
Adult heart			+
Adrenal gland	+		+
Muscle	+		+
Lung	+		
Testis	+		
Ovary	+		
Prostate	+		
Thyroid	+		
Kidney			+
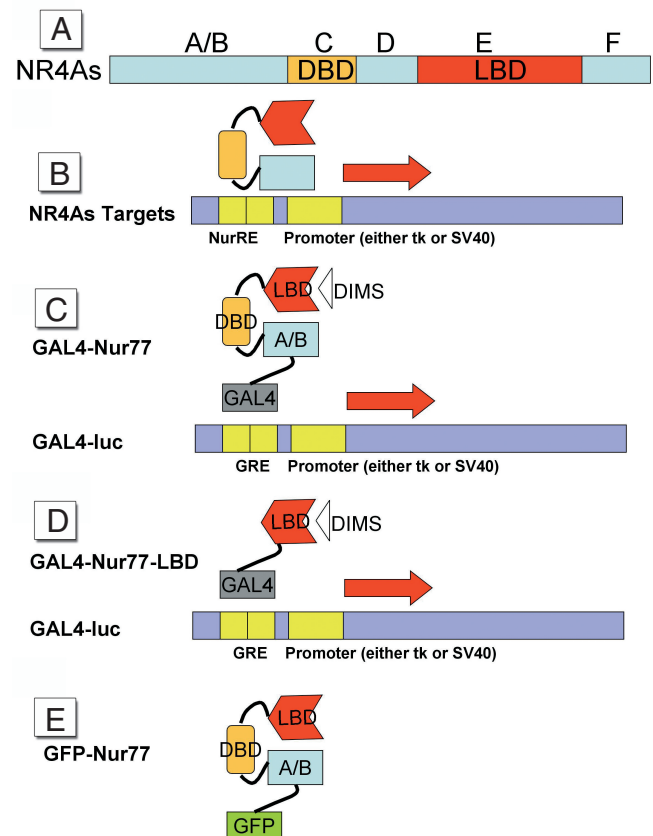
Fig. 1. Domains structures and reporter assays. **A.** Domain structure for NR4A1 family protein is shown. **B.** NurRE-luciferase. Nur77-response element (NurRE) is cloned in front the TK-luciferase construct. Binding of NR4A1/2/3 will activate reporter expression. **C, D.** Gal4/GRE-luciferase and GAL4/DBD-NR4A1 (C)/LBD **(D)**. The reporter is constructed by cloning the GAL response elements (GRE) in front of the TK-luciferase constructs. GAL4 DNA binding domain fused either with full-length Nur77 **(C)** or Nur77 ligand binding domain **(D)** Binding of the GAL4-Nur77 or GAL4-Nur77LBD will activate the reporter expression. **E.** GFP-TR3.

survival function of NR4A1 has been demonstrated by both gene over-expression and silencing studies for both the extrinsic and intrinsic apoptotic pathways.

Pro-apoptotic role of NR4A1

Interestingly, NR4A1 can also mediate an opposite biological effect, namely a pro-apoptotic effect. NR4A1 was reported to be pro-apoptotic in thymocytes (Zhang et al., 1999; Winoto and Littman, 2002). In self-reactive immature thymocytes, NR4A1 expression was induced and increased apoptosis was observed following stimulation of the T cell receptor (TCR). Expression of a dominant negative NR4A1 mutant or antisense blocked the TCR dependent apoptosis. Over-expression of NR4A1 in transgenic mice resulted in high levels of apoptosis in thymocytes (Calnan et al., 1995), confirming its pro-apoptosis role *in vivo* and thus its physiological relevance. Interestingly, when knockout animals were generated, no phenotype was observed (Lee et al., 1995), suggesting the likely redundancy of gene functions, e.g. with NR4A3 (see below). It is worth noting that the above described opposing pro-apoptotic and anti-apoptotic effects can even occur within the same cell system, e.g. NCI-H460 (Li et al., 1998; Kolluri et al., 2003) depending on the context (e.g. specific apoptotic stimuli) and the localization of NR4A1. However, the exact regulation of the two opposing effects is still to be elucidated.

The pro-apoptosis role of NR4A1 may be achieved by the transactivation function of NR4A1 as a transcription factor by targeting genes responsible for promoting apoptosis, e.g. FasL or TRAIL (Zhang et al., 1999; Chintharlapalli et al., 2005). Thus, this function is associated with nuclear localization of NR4A1. However, studies also revealed a unique mechanism in which NR4A1 translocates to the mitochondria and binds to Bcl-2 to form a pro-apoptosis complex in response to apoptotic stimuli. This triggers cytochrome C release and apoptosis as demonstrated in LNCaP human prostate and other cancer cells (Li et al., 2000). Domain analysis indicated that the LBD is responsible for Bcl-2 binding. This binding results in the conformational change of Bcl-2, which subsequently leads to NR4A1 mitochondrial localization (Lin et al., 2004), although NR4A1 lacks classic mitochondria-targeting sequences. The presence of three putative nuclear export sequences (NESs) in the LBD is probably responsible for the translocation out of the nucleus. These events cause activation of Apaf-1 and caspase 9 followed by cleavage of caspase 3 and apoptosis.

Although the mechanisms of regulation of NR4A1 activation is not completely understood, the phosphorylation status of NR4A1 appears to play an important role in its transcriptional activity. Phosphorylation, which can be carried out by Akt or the MAPK family kinases, results in predominantly cytoplasmic localization of the protein (Hsu et al., 2004). The extent of phosphorylation depends on the nature of the stimuli, resulting in differential regulation of its transcriptional activity. Nerve growth factor (NGF) probably induces NR4A1 expression and phosphorylation at Ser350 in the DBD, which impairs its binding to NBRE (Hirata et al., 1993; Katagiri et al., 1997). Akt kinase is also shown to target Ser316 in a PI-3K-dependent manner, thus inhibiting DNA binding and gene activation, resulting in suppression of NGFI-B-induced apoptosis in fibroblasts and TCR-mediated cell death in T-cell hybridomas (Masuyama et al., 2001; Pekarsky et al., 2001). NGF also induces additional phosphorylation at Ser105 through the Trk/Ras/MAPK pathway, resulting in nuclear export of NR4A1 (Katagiri et al., 2000) along with RXR via heterodimerization. In contrast, dephosphorylation of Ser316 through adrenocorticotropin (ACTH) treatment in adrenal-derived Y1 cells allows DNA binding of NR4A1 (Davis and Lau, 1994; Li and Lau, 1997).

NR4A2

In contrast to NR4A1, only pro-survival effects, but not pro-apoptotic effects, have been reported for NR4A2. NR4A2 is highly expressed in brain tissues (Table 2), with lower expression in some several other tissues. NR4A2 knockout mice were impaired in development and maintenance of midbrain dopaminergic neurons, and they die shortly after birth (Law et al., 1992; Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998). Heterozygous mice (NR4A2 +/-) appear healthy, but were more susceptible to the action of neurotoxins (Le et al., 1999). NR4A2 has also been linked to Parkinson's disease, as mutations in

Table 3. Mouse transgenic and knockout phenotypes.

ORPHAN RECEPTORS	TRANSGENIC PHENOTYPES	KNOCKOUT PHENOTYPES	REFERENCES
NR4A1	Massive thymocyte apoptosis	Minimal phenotype	(Lee et al., 1995; Cheng et al., 1997)
NR4A2	NT	Development lethal (die right after birth); lack dopamine-producing neuron	(Zetterstrom et al., 1997)
NR4A3	NT	Embryonic lethal; defect in proliferation of the semicircular canals of the mouse inner ear	(Ponnio et al., 2002; DeYoung et al., 2003)

NT: not tested.

the NR4A2 gene were recently found in certain patients (Le et al., 2003). Over-expression of NR4A2 increased the resistance of neural stem cells to neurotoxins 6-hydroxy-DA and MPTP (Lee et al., 2002). Together, these findings point towards a cyto-protective function for NR4A2 in the CNS.

Pro-survival/anti-apoptotic properties have also been observed for NR4A2 using a transient siRNA transduction approach and a lentiviral NR4A2-siRNA vector in several other cancer cell lines of different origin, similar to that of NR4A1. In these experiments, silencing of NR4A2 caused apoptosis assessed by TUNNEL-based assays (Table 3) (Ke et al., 2004). However, stable NR4A2-silenced cancer cells can be generated for HeLa cells, PC3 and HCT116 cells and these stably transduced cells displayed greatly reduced anchorage-independent growth, with minimal reduction in anchorage-dependent growth, as measured by cloning efficiency assays. Further experiments indicate that this reduced anchorage-independent growth was largely due to increased anoikis (apoptosis caused by cell detachment). Since anchorage-independent growth is a hallmark of cell transformation, this result strongly suggests a role for NR4A2 in cell transformation. This was further confirmed by the failure of cancer cells transduced with the NR4A2 siRNA vector to establish tumor in the xenograft model (Ke et al, unpublished).

NR4A3

NR4A3 has been shown to be functionally redundant with NR4A1 in T-cell apoptosis (Cheng et al., 1997). The tissue expression pattern of NR4A3 is also similar to that of NR4A1 (Cheng et al., 1997) (Table 2). On the other hand, our unpublished data also indicate a pro-survival role of NR4A3 (Ke and Sundaram et al., unpublished observations). Our observations are further supported by other studies in which NR4A3 knockout mice were shown to be embryonic lethal (DeYoung et al., 2003), similar to NR4A2 knockout mice, but in contrast to NR4A1 knockout mice (no phenotype), These results also suggest that NR4A1 and NR4A3 have non-overlapping functions as well.

In summary, all three members of Nurr77 receptor family seem to have pro-survival function in certain

contexts, while NR4A1 and NR4A3 also have pro-apoptotic functions in other contexts.

### NR4A1/2/3 and potential roles in human cancers

Earlier reports have described the possible involvement of NR4A1, 2, and 3 in a variety of human diseases (Le et al., 2003; Hsu et al., 2004), including NR4A1 in inflammatory disease, allergy and atherosclerosis, and NR4A2 in Parkinson disease and rheumatoid arthritis (McEvoy et al., 2002). However, the accumulated data also implicate this family of receptors in cell transformation and human carcinogenesis. A strong evidence for the role of the Nur77/NGFIB subfamily of nuclear hormone receptors in cell transformation is the observation that all three members are down-regulated in non-transformed HeLaHF revertant cells as compared to the transformed parental HeLa cells (Fig. 2) (Boylan et al., 1996; Ke et al., 2004; Li et al., 2005). HeLa is a cervical cancer cell line exhibiting typical transformed phenotypes, including anchorage-independent growth *in vitro* and tumorigenisis *in vivo*. HeLaHF is a revertant variant isolated from HeLa cells following exposure to the mutagen ethane methyl sulfonate. Interestingly, when HF cells were re-transformed and became tumorigenic, all members of this family were also up-regulated. Conversely, silencing of these genes in HeLa cells caused a reversal of cell transformation (see above), confirming the causal roles of these genes in cell transformation. Our recent unpublished data that the silencing of NR4A2 causes a drastic reduction in tumorigenicity of PC3 prostate cancer model further underscores the importance of this receptor for cell transformation and tumorigenicity (Ke et al., unpublished).

The second evidence that NR4A1 is involved in cancer came from the observation that a 270 fold-enhanced transcription of NR4A1 occurs in extraskeletal

Table 4. Anti-apoptotic and pro-apoptotic properties in cell lines tested.

	NR4A1	NR4A2	NR4A3
Cervical cancer HeLa cell	+	+	+
Colon cancer DLD1	+	+	
Colon cancer HCT116	+	+	
Prostate cancer PC3	+	+	
Glioma line U87	+	+	
Pancreatic cancer line AsPC1	+	+	

+: anti-apoptotic effect, -: proapoptotic effect.
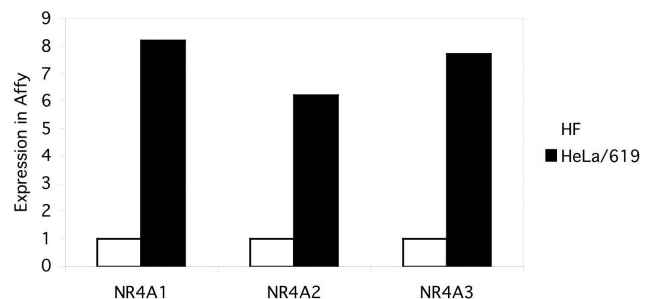
Fig. 2. NR4A1/2/3 expression in HeLa, its non-transformed derivative HeLa/HF, and retransformed derivative HeLa/HF/Rz619. Expression of NR4A1, NR4A2, and NR4A3 is determined by Real-time RT-PCR (Taqman). Expression is normalized to the expression level in HeLa/HF cells.

myxoid chondrosarcoma due to a chromosomal translocation (Labelle et al., 1999). There is also a paradoxical association of high levels of Bcl-2 with favorable clinical outcome for patients with several types of cancers, including breast, colon, and NSCL cancers. The observation that NR4A1 binds Bcl-2 may provide a possible mechanism for the above observation, since NR4A1 binding to Bcl-2 induces apoptosis (Lin et al., 2004).

NR4A1/2/-3 as potential drug targets for human diseases

The cumulative information suggests that the Nur77/NGFIB family of nuclear hormone receptors may share similar pro-survival functions at least in certain cancer cells, and thus these properties could potentially be explored to develop antagonist drugs for cancer. Since RNAi mimics antagonist activity, our *in vivo* RNAi experiment with NR4A2 suggests that this receptor can serve as a drug target provided that antagonists can be identified. On the other hand, the pro-apoptosis nature of NR4A1 can also be explored to develop agonist therapeutics (Chintharlapalli et al., 2005).

The absence of a conventional ligand-binding pocket raises uncertainty for identifying modulators for the functions of these receptors. However, a recent study identified a novel coactivator interaction site between helices 11 and 12 for both NR4A2 and NR4A1, instead of the classical coactivator interaction site in the E/F domain (helix 12) (Codina et al., 2004). In addition, Chintarlapalli and colleagues identified an NR4A1-binding agonist, which demonstrates the possibility of activity-modulators for this family of receptors (Chintharlapalli et al., 2005). This study described that 1,1-bis (3'-indolyl)-1-(p-substituted phenyl) methane-containing trifluoromethyl, hydrogen and methoxy substituents (also called selected C-substituted DIMs (3,3'-diindolylmethanes)) induced NR4A1-dependent transactivation in cancer cell lines (e.g. Panc28 pancreatic cancer cell line) and apoptosis. These active compounds were identified by screening based on GAL4-Nur77/GAL4 reporter assay, in which a structure-dependent activation of NR4A1 was monitored in cells co-transfected with GAL4-Nur77 (full-length) chimera, or GAL4-Nur77 (E/F)(LBD or E/F domain) chimera, along with a reporter vector containing five GAL4 response elements linked to luciferase gene (Fig. 1C, D). The activities of these compounds were also confirmed in reporter assays using NurRE (Nur response element)-reporter vector (Fig. 1B), suggesting these compounds not only bind to NR4A1 via its LBD but also activate NR4A1 mediated transcription. Particularly, binding of these compounds is very important for interaction between NR4A1 and a few co-activators such as SRC-1, PGC-1 and TRAP220. Therefore, these C-substituted DIMs are a novel class of compounds that induce E/F domain-dependent activation of NR4A1. Furthermore, these NR4A1 agonists also reduce Panc28 cell survival and induce apoptosis. These biological effects do not involve the translocation of NR4A1 to the mitochondria, or changes in levels of BAX and Bcl-2. However, TRAIL expression seems to be activated, suggesting transcriptional activation of the down-stream targets of NR4A1. In addition, when these compounds were tested in the Panc28 xenograft tumor models *in vivo*, anti-tumor activity was observed with apparently little toxicity. These results demonstrate for the first time the potential of the anti-cancer utility of the NR4A1 agonists.

The identification of functional ligands for NR4A1 indicates that it may also be possible to find functional ligands for both NR4A2 and NR4A3 owing to the existence of similar coactivator interfaces on all three receptors. The successful identification of the functional binders for NR4A1 using GAL4-Nur77 (full-length or E/F)-luc suggests that a similar reporter assay can be easily developed to identify functional binders for NR4A2 and NR4A3.

Stable silencing of NR4A2 led to the reversal of cell transformation and reduction in tumorigenicity (Ke et al., 2004; Ke et al., unpublished data). Although NR4A2 is less extensively studied than NR4A1, it may be a preferable anti-cancer target to NR4A1. This is based on several assumptions: 1) unlike NR4A1, so far NR4A2 has not yet been shown to have pro-apoptosis effects; 2) NR4A2 has a unique role in anchorage-independent growth, which is one of the hallmarks of cell transformation. In other words, NR4A2 may serve as a transformation-specific target with potentially less toxicity than targets also involved in apoptotic functions; 3) Since the expression of NR4A2 is restricted to the brain and CNS among normal tissues, the potential toxicity of targeting NR4A2 could be limited, especially for compounds that do not pass through the blood-brain barrier.

In summary, it is clear that NR4A1, NR4A2 and NR4A3 orphan receptors play different roles in cell transformation. These properties can potentially be utilized as targeted anti-cancer therapy. A new class of agonists against NR4A1 has been identified for this utility, which can serve as a paradigm for the search of antagonists for other members of this unique receptor family.

References

Baker K.D., Shewchuk L.M., Kozlova T., Makishima M., Hassell A., Wisely B., Caravella J.A., Lambert M.H., Reinking J.L. and Krause H. (2003). The Drosophila orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. Cell 113, 731-742.

Boylan M.O., Athanassiou M., Houle B., Wang Y. and Zarbl H. (1996). Activation of tumor suppressor genes in nontumorigenic revertants of the HeLa cervical carcinoma cell line. Cell Growth Differ 7, 725-

735.

Bras A., Albar J.P., Leonardo E., de Buitrago G.G. and Martinez A.C. (2000). Ceramide-induced cell death is independent of the Fas/Fas ligand pathway and is prevented by Nur77 overexpression in A20 B cells. Cell. Death Differ. 7, 262-271.

Calnan B.J., Szychowski S., Chan F.K., Cado D. and Winoto A. (1995). A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. Immunity 3, 273-282.

Castillo S.O., Baffi J.S., Palkovits M., Goldstein D.S., Kopin I.J., Witta J., Magnuson M.A., and Nikodem V.M. (1998). Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. Mol. Cell Neurosci. 11, 36-46.

Cheng L.E., Chan F.K., Cado D. and Winoto A. (1997). Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. EMBO J. 16, 1865-1875.

Chintharlapalli S., Burghardt R., Papineni S., Ramaiah S., Yoon K. and Safe S. (2005). Activation of Nur77 by selected 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes induces apoptosis through nuclear pathways. J. Biol. Chem. 280, 24903-24914.

Codina A., Benoit G., Gooch J.T., Neuhaus D., Perlmann T. and Schwabe J.W. (2004). Identification of a novel co-regulator interaction surface on the ligand binding domain of Nurr1 using NMR footprinting. J. Biol. Chem. 279, 53338-53345.

Davis I.J. and Lau L.F. (1994). Endocrine and neurogenic regulation of the orphan nuclear receptors Nur77 and Nurr-1 in the adrenal glands. Mol. Cell Biol. 14, 3469-3483.

DeYoung R.A., Baker J.C., Cado D. and Winoto A. (2003). The orphan steroid receptor Nur77 family member Nor-1 is essential for early mouse embryogenesis. J. Biol. Chem .278, 47104-47109.

Enmark E. and Gustafsson J.A. (1996). Orphan nuclear receptors--the first eight years. Mol. Endocrinol. 10, 1293-1307.

Fernandez P.M., Brunel F., Jimenez M.A., Saez J.M., Cereghini S. and Zakin M.M. (2000). Nuclear receptors Nor1 and NGFI-B/Nur77 play similar, albeit distinct, roles in the hypothalamo-pituitary-adrenal axis. Endocrinology 141, 2392-2400.

Forman B.M., Umesono K., Chen J. and Evans R.M. (1995). Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell 81, 541-550.

Hirata Y., Kiuchi K., Chen H.C., Milbrandt J. and Guroff G. (1993). The phosphorylation and DNA binding of the DNA-binding domain of the orphan nuclear receptor NGFI-B. J. Biol. Chem. 268, 24808-24812.

Hsu H.C., Zhou T. and Mountz J.D. (2004). Nur77 family of nuclear hormone receptors. Curr. Drug Targets Inflamm. Allergy 3, 413-423.

Katagiri Y., Hirata Y., Milbrandt J. and Guroff G. (1997). Differential regulation of the transcriptional activity of the orphan nuclear receptor NGFI-B by membrane depolarization and nerve growth factor. J. Biol. Chem. 272, 31278-31284.

Katagiri Y., Takeda K., Yu Z.X., Ferrans V.J., Ozato K. and Guroff G. (2000). Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B. Nat. Cell Biol .2, 435-440.

Ke N., Claassen G., Yu D.H., Albers A., Fan W., Tan P., Grifman M., Hu X., Defife K. and Nguy V. (2004). Nuclear hormone receptor NR4A2 is involved in cell transformation and apoptosis. Cancer Res. 64, 8208-8212.

Kolluri S.K., Bruey-Sedano N., Cao X., Lin B., Lin F., Han Y.H., Dawson M.I. and Zhang X.K. (2003). Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells. Mol. Cell Biol. 23,

8651-8667.

Labelle Y., Bussieres J., Courjal F. and Goldring M.B. (1999). The EWS/TEC fusion protein encoded by the t(9;22) chromosomal translocation in human chondrosarcomas is a highly potent transcriptional activator. Oncogene 18, 3303-3308.

Law S.W., Conneely O.M., DeMayo F.J. and O'Malley B.W. (1992). Identification of a new brain-specific transcription factor, NURR1. Mol. Endocrinol. 6, 2129-2135.

Le W., Conneely O.M., He Y., Jankovic J. and Appel S.H. (1999). Reduced Nurr1 expression increases the vulnerability of mesencephalic dopamine neurons to MPTP-induced injury. J. Neurochem. 73, 2218-2221.

Le W.D., Xu P., Jankovic J., Jiang H., Appel S.H., Smith R.G. and Vassilatis D.K. (2003). Mutations in NR4A2 associated with familial Parkinson disease. Nat. Genet. 33, 85-89.

Lee M.A., Lee H.S., Cho K.G., Jin B.K., Sohn S., Lee Y.S., Ichinose H., and Kim S.U. (2002). Overexpression of midbrain-specific transcription factor Nurr1 modifies susceptibility of mouse neural stem cells to neurotoxins. Neurosci. Lett. 333, 74-78.

Lee S.L., Wesselschmidt R.L., Linette G.P., Kanagawa O., Russell J.H., and Milbrandt J. (1995). Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). Science 269, 532-535.

Li H., Kolluri S.K., Gu J., Dawson M.I., Cao X., Hobbs P.D., Lin B., Chen G., Lu J. and Lin F. (2000). Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. Science 289, 1159-1164.

Li Q.X., Tan P., Ke N. and Wong-Staal F. (2005). Ribozyme technology and its application in cancer gene target identification and validation. In: Genomics in cancer drug development. Hampton G. and Silkora K. (eds). Academic Press (in press).

Li Y. and Lau L.F. (1997). Adrenocorticotropic hormone regulates the activities of the orphan nuclear receptor Nur77 through modulation of phosphorylation. Endocrinology 138, 4138-4146.

Li Y., Lin B., Agadir A., Liu R., Dawson M.I., Reed J.C., Fontana J.A., Bost F., Hobbs P D. and Zheng Y. (1998). Molecular determinants of AHPN (CD437)-induced growth arrest and apoptosis in human lung cancer cell lines. Mol. Cell Biol. 18, 4719-4731.

Lin B., Kolluri S.K., Lin F., Liu W., Han Y.H., Cao X., Dawson M.I., Reed J.C. and Zhang X.K. (2004). Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. Cell 116, 527-540.

Maira M., Martens C., Philips A. and Drouin J. (1999). Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. Mol. Cell Biol. 19, 7549-7557.

Mangelsdorf D.J., Thummel C., Beato M., Herrlich P., Schutz G., Umesono K., Blumberg B., Kastner P., Mark M., Chambon P. and Evans R.M. (1995). The nuclear receptor superfamily: the second decade. Cell 83, 835-839.

Masuyama N., Oishi K., Mori Y., Ueno T., Takahama Y. and Gotoh Y. (2001). Akt inhibits the orphan nuclear receptor Nur77 and T-cell apoptosis. J. Biol. Chem. 276, 32799-32805.

McEvoy A.N., Murphy E.A., Ponnio T., Conneely O.M., Bresnihan B., FitzGerald O. and Murphy E.P. (2002). Activation of nuclear orphan receptor NURR1 transcription by NF-kappa B and cyclic adenosine 5'-monophosphate response element-binding protein in rheumatoid arthritis synovial tissue. J. Immunol. 168, 2979-2987.

Meinke G. and Sigler P.B. (1999). DNA-binding mechanism of the

monomeric orphan nuclear receptor NGFI-B. Nature Struct. Biol. 6, 471-477.

Paulsen R.F., Granas K., Johnsen H., Rolseth V. and Sterri S. (1995). Three related brain nuclear receptors, NGFI-B, Nurr1, and NOR-1, as transcriptional activators. J. Mol. Neurosci. 6, 249-255.

Pekarsky Y., Hallas C., Palamarchuk A., Koval A., Bullrich F., Hirata Y., Bichi R., Letofsky J. and Croce C.M. (2001). Akt phosphorylates and regulates the orphan nuclear receptor Nur77. Proc. Natl. Acad. Sci. USA 98, 3690-3694.

Perlmann T. and Jansson L. (1995). A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev. 9, 769-782.

Ponnio T., Burton Q., Pereira F.A., Wu D.K. and Conneely O M. (2002). The nuclear receptor Nor-1 is essential for proliferation of the semicircular canals of the mouse inner ear. Mol. Cell Biol. 22, 935-945.

Saucedo-Cardenas O., Quintana-Hau J.D., Le W.D., Smidt M.P., Cox J. J., De Mayo F., Burbach J.P. and Conneely O.M. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc. Natl. Acad. Sci. USA 95, 4013-4018.

Suzuki S., Suzuk N., Mirtsos C., Horacek T., Lye E., Noh S.K., Ho A., Bouchard D., Mak T.W. and Yeh W.C. (2003). Nur77 as a survival factor in tumor necrosis factor signaling. Proc. Natl. Acad. Sci. USA 100, 8276-8280.

Wang Z., Benoit G., Liu J., Prasad S., Aarnisalo P., Liu X., Xu H., Walker N.P. and Perlmann T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature 423, 555-560.

Weih F., Ryseck R.P., Chen L. and Bravo R. (1996). Apoptosis of nur77/N10-transgenic thymocytes involves the Fas/Fas ligand pathway. Proc. Natl. Acad. Sci. USA 93, 5533-5538.

Wilson T.E., Fahrner T.J., Johnston M. and Milbrandt J. (1991). Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science 252, 1296-1300.

Winoto A. and Littman D.R. (2002). Nuclear hormone receptors in T lymphocytes. Cell 109 Suppl, S57-66.

Wu Q., Li Y., Liu R., Agadir A., Lee M.O., Liu Y. and Zhang X. (1997). Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization. EMBO J. 16, 1656-1669.

Zetterstrom R.H., Solomin L., Jansson L., Hoffer B.J., Olson L. and Perlmann T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. Science 276, 248-250.

Zetterstrom R.H., Solomin L., Mitsiadis T., Olson L. and Perlmann T. (1996). Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. Mol. Endocrinol. 10, 1656-1666.

Zhang J., DeYoung A., Kasler H.G., Kabra N.H., Kuang A.A., Diehl G., Sohn S.J., Bishop C. and Winoto A. (1999). Receptor-mediated apoptosis in T lymphocytes. Cold Spring Harb. Symp. Quant. Biol. 64, 363-371.